Renal brush-border-membrane vesicles prepared from newborn rats by free-flow electrophoresis and their proline uptake

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A method for the isolation of brush-border membranes from newborn-rat kidney, employing centrifugation and free-flow electrophoresis, is described. The composition and purity of the preparation was assessed by determination of enzyme activities specific for various cellular membranes. Free-flow electrophoresis resolves the newborn-rat renal membrane suspension into two populations of alkaline phosphatase-enriched brush-border membranes, designated 'A' and 'B', with the A peak also showing activity of ($\text{Na}^+ + \text{K}^+$)-stimulated ATPase, the basolateral membrane marker enzyme, whereas those of the B peak were enriched 11-fold in alkaline phosphatase and substantially decreased in ($\text{Na}^+ + \text{K}^+$)-stimulated ATPase activity. Membranes in the A peak showed a 7-fold enrichment of alkaline phosphatase, and ($\text{Na}^+ + \text{K}^+$)-stimulated ATPase activity similar to that of the original homogenate. Proline uptake employed to assess osmotic dependency revealed 7% binding of proline to the B vesicles and 31% to the A vesicles. This contrasts with 60% proline binding to vesicles prepared by centrifugation alone. Unlike vesicles from adult animals, proline uptake by B vesicles did not show an Na^+ -stimulated overshoot, but did exhibit an Na^+ -gradient enhanced rate of early proline entry.

Developmental studies *in vitro* of rat renal tubular-membrane transport of amino acids have been carried out by using the cortical slice (Baerlocher *et al.*, 1970, 1971; Reynolds & Segal, 1976; Reynolds *et al.*, 1978) and isolated renal tubule models (Segal *et al.*, 1971; Roth *et al.*, 1977). Data obtained from these models have not always been consistent and their interpretation has been uniformly hindered by the difficulties inherent in the study of bipolar transport in renal tubular epithelial cells. Methods for preparation of brush-border-membrane vesicles (Booth & Kenny, 1974) from adult rat kidney have enabled investigators to circumvent this problem in the study of amino acid (McNamara *et al.*, 1976), organic acid (Roth *et al.*, 1982), sugar (Aronson & Sacktor, 1975) and ion (Kinsella & Aronson, 1980) transport. However, application of these methods to study of newborn-rat transport phenomena has not been widely made.

In two previous reports, Goldmann and his

co-workers isolated and characterized a brush-border-vesicle preparation from newborn-rat kidney (Goldmann *et al.*, 1976), and studied proline uptake by these vesicles (Goldmann *et al.*, 1979). In the latter report the uptake of proline by brush-border vesicles from newborn-rat kidney differed markedly from that of the adult. Whereas an Na^+ -stimulated overshoot is observed in adult-rat vesicles, newborn-rat vesicles under similar conditions did not exhibit an overshoot and proline uptake continued to increase in an almost linear fashion up to 60 min without reaching a steady state. Although an osmotic-dependency experiment indicated little proline binding at 2 min (Goldmann *et al.*, 1979), the pattern of uptake at later incubation times suggested the possibility that significant binding might be occurring. In subsequent experiments reported in the present paper uptake measured at 20 min indicated considerable binding of proline to the newborn-rat brush-border membranes. This led us to assume that the renal brush-border vesicles of newborn rats prepared by differential centrifugation (Goldmann *et al.*, 1976) may be more diverse than those of adult membranes derived by the same procedure. We

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therefore chose to utilize homogenization followed by free-flow electrophoresis to prepare renal brush-border vesicles from newborn rats. Herein we describe the finding of two populations of brush-border membranes from such animals and examine the uptake of proline.

Materials and methods

Membrane preparation

Renal cortical plasma membranes were isolated by differential centrifugation by using a modification of the method described by Heidrich *et al.* (1972), shown in Fig. 1. Twelve litters of Sprague-Dawley rats, up to 24 h old, were killed by decapitation, the kidneys removed and placed in a Petri dish on ice. The kidneys were homogenized (1:2, w/v) in buffer I (0.25 M-sucrose in 0.01 M-triethanolamine hydrochloride titrated to pH 7.4 with NaOH) by using four strokes of a Dounce homogenizer (tight pestle) and three strokes in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle [0.01 inch

Fig. 1. Scheme of the homogenization and centrifugation of newborn-rat renal tissue before free-flow electrophoresis

The final product is then separated by free-flow electrophoresis, as described in the text.

(0.254 mm) clearance] at 300 rev./min. All operations thereafter were performed at 4°C. The homogenate was centrifuged at 700 g for 10 min. The fluffy upper pellet was resuspended in the supernatant and centrifuged at 700 g for 10 min. The fluffy upper pellet was again suspended in the supernatant and centrifuged at 16 000 g for 20 min. The fluffy upper pellet was then suspended in the supernatant and homogenized with three strokes of a motor-driven Potter-Elvehjem homogenizer, and centrifuged at 16 000 g for 20 min. The resultant supernatant was discarded and the fluffy upper pellet was resuspended in half the original volume of buffer I, and homogenized with three strokes of a motor-driven Potter-Elvehjem homogenizer. An equal volume of buffer I was added to this suspension, which was centrifuged at 16 000 g for 20 min. The resultant fluffy white upper pellet was suspended in 4 ml of electrophoresis running buffer (0.25 M-sucrose in 8.5 mM-triethanolamine titrated to pH 7.4 with acetic acid) and centrifuged at 500 g for 10 min. The resultant fluffy white upper layer was resuspended in the supernatant to give a protein concentration of 4–6 mg/ml and injected into a Bender and Hobein (Munich, Germany) VaP5 free-flow electrophoresis apparatus through the port above fraction 70 at a rate of 5.7 ml/h. During the free-flow run, the voltage was maintained at 875 V and the temperature was maintained at 4.5°C, conditions similar to those previously reported (Reynolds *et al.*, 1980). Most free-flow electrophoresis machines are designed to maintain a constant current and allow voltage to vary inversely with the resistivity of the injected material. In this method, the current was adjusted manually throughout the run to maintain a constant voltage in order to maintain a straight path of travel of the membranes through the separation chamber. In our standard runs, an average of 3 ml of membrane suspension was injected. Running buffer flowed through the separation chamber at a rate of 150 ml/h. The buffer bathing the electrodes consisted of 85 mM-triethanolamine titrated to pH 7.4 with acetic acid. After separation, the samples were assayed for appropriate membrane enzyme markers and protein concentration. Fractions under the two peaks for alkaline phosphatase, the marker enzyme for luminal membranes (Heidrich *et al.*, 1972), were pooled and collected by centrifugation at 32 000 g for 20 min. The membranes were resuspended in buffer II {100 mM-mannitol, 2 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], titrated to pH 7.4 by the addition of Tris} to a final protein concentration of about 0.1 mg/ml, as determined by the method of Lowry *et al.* (1951).

Enzyme determinations

All materials used were of the highest quality available. Enzyme and protein determinations were

performed on portions of the initial renal homogenate, free-flow electrophoresis outflow fractions and pooled membrane fractions after electrophoresis. These determinations were performed according to standard procedures: alkaline phosphatase (EC 3.1.3.1) and acid phosphatase (EC 3.1.3.2) (Sigma Technical Bulletin, 1963); succinate dehydrogenase (EC 1.3.99.1) (Pennington, 1961); and $(\text{Na}^+ + \text{K}^+)$ -stimulated ATPase (EC 3.6.1.3) (Ismail-Beigi & Edelman, 1971). Glucose 6-phosphatase (EC 3.1.3.9) activity was measured in an assay modified from that of Hubscher & West (1965). Glucose 6-phosphate (0.2M; 0.1ml), 0.5ml of 0.05M-maleate buffer containing 4mM-EDTA, pH 6.0, and 0.1ml of membrane suspension were incubated together for 15 min at 37°C. The reaction was stopped by adding 1.0ml of trichloroacetic acid to precipitate protein. After centrifugation, 1.0ml of the supernatant was used for determination of P_i as previously described (Kinsolving *et al.*, 1963).

Electron microscopy

Suspensions of pooled free-flow electrophoresis outflow fractions corresponding to the 'A' and 'B' peaks were examined by electron microscopy. Negatively stained wet preparations were made by placing a drop of membrane suspension on a carbon-coated Formvar grid, adding one drop of 4% phosphotungstic acid, pH 7.0, and examining the preparations in a Philips 300 electron microscope.

Transport measurements into brush-border-membrane vesicles

The measurement of L-proline uptake by membrane vesicles using Millipore filtration on HAWP filters (0.45 μm) was performed by the technique described by McNamara *et al.* (1976). All uptake experiments were performed at 22°C.

Materials

Radioactive compounds were purchased from New England Nuclear Corp: $[\text{U}-^{14}\text{C}]$ proline (sp. radioactivity 261 Ci/mol) and $\text{L}-[\text{U}-^3\text{H}]$ glucose (sp. radioactivity 17.46 Ci/mmol). All other chemicals were obtained from commercial sources and were of the highest purity available.

Results

Free-flow electrophoresis separated the renal membranes into two populations that migrated at different rates in the separation chamber owing to membrane surface charge and size (Hannig *et al.*, 1975). Luminal and antiluminal marker enzyme determinations were made on sequential free-flow electrophoresis fractions, and the results are shown in Fig. 2(a). Alkaline phosphatase activity was employed as the luminal marker enzyme and $(\text{Na}^+ + \text{K}^+)$ stimulated ATPase as the antiluminal marker enzyme. Fractions 18–36 (referred to as the

Fig. 2. Separation of isolated renal cell membranes by free-flow electrophoresis

After homogenization and centrifugation, isolated renal cellular membranes were separated by using a VaP5 free-flow electrophoresis apparatus as described in the text. The left side of the separation chamber is positively charged (+) and the right side negatively charged (–). The enzymic activities of $(\text{Na}^+ + \text{K}^+)$ -stimulated ATPase (○) and alkaline phosphatase (●) were determined in the fractions and are shown in (a). The profile of protein concentration in the corresponding fractions is shown in (b). (b) represents the profiles of one free-flow electrophoretic run with duplicate determinations for each point shown. At least four separate preparations showed profiles similar to that shown here.

'A' peak) appeared to contain a mixture of both brush-border and basolateral membranes and fractions 38–48 (referred to as the 'B' peak) contained primarily brush-border membranes. The profile of protein concentration in the corresponding outflow fractions is shown in Fig. 2(b). Protein concentration of free-flow electrophoresis fractions 18–48 ranged between 0.7 and 1.0mg/ml, and were relatively constant in these fractions.

To characterize these membranes further, free-flow-electrophoresis fractions 18–36 and 38–48 were each pooled and the specific activities of five marker enzymes were determined in these pooled membrane fractions as well as in the initial renal homogenate. The results of these determinations are shown in Table 1. The specific activity of alkaline phosphatase in both pooled fractions corresponding to the A and B peaks show considerable enrichment when compared with the original homogenate, which is consistent with the presence of brush-border membranes. The specific activity of this enzyme in the B peak was almost 2-fold that in the A peak. The specific activity of $(\text{Na}^+ + \text{K}^+)$ -stimulated ATPase,

Table 1. *Specific activities of enzymes in original homogenate and A and B peaks, prepared from newborn-rat kidneys*
Renal membranes were isolated and assays were performed as described in the Materials and methods section. Values shown are means $\pm$ S.E.M. for at least three experiments performed in duplicate. A/H and B/H are the ratios of activity in the A and B peaks respectively to activity in homogenate.

Enzymes	Cellular marker	Homogenate	A peak	A/H	B peak	B/H
(Na ⁺ + K ⁺)-stimulated ATPase (μ mol of P _i liberated/mg per h)	Basolateral membrane	2.24 $\pm$ 0.51	1.96 $\pm$ 0.23	0.88	0.11 $\pm$ 0.06	0.05
Mg ²⁺ -dependent ATPase (μ mol of P _i liberated/mg per h)		6.52 $\pm$ 0.31	12.96 $\pm$ 0.50	1.98	7.64 $\pm$ 0.45	1.17
Alkaline phosphatase (units/mg per h)	Brush-border membrane	59.51 $\pm$ 1.00	400.41 $\pm$ 31.6	6.73	687.83 $\pm$ 19.51	11.56
Acid phosphatase (units/mg per h)	Lysosomes	147.62 $\pm$ 10.70	23.79 $\pm$ 7.41	0.16	55.44 $\pm$ 6.61	0.38
Glucose 6-phosphatase (mmol of P _i liberated/mg per h)	Microsomes	4.43 $\pm$ 0.16	5.09 $\pm$ 0.40	1.15	7.93 $\pm$ 0.37	1.79
Succinate dehydrogenase (<i>A</i> ₄₉₀ /mg per h)	Mitochondria	6.95 $\pm$ 0.46	2.81 $\pm$ 0.48	0.40	0.58 $\pm$ 0.14	0.08

the basolateral membrane marker, in the A peak was the same as in the initial homogenate, whereas in the B peak it was only 5% that of the initial homogenate. The specific activities of acid phosphatase, succinate dehydrogenase and glucose 6-phosphatase, the marker enzymes for lysosomes, mitochondria and endoplasmic reticulum respectively, are also shown in Table 1. These data indicate that the B fraction is a highly purified population of brush-border membranes.

The pooling of free-flow fractions 18–36, corresponding to the A peak, and 38–48, corresponding to the B peak, yielded approx. 2.0–2.5 mg and 1.0–1.5 mg respectively of renal membranes per 10 g of newborn-rat kidney. This yield is obtained after 2.5 h of preparative homogenization and centrifugation, and a 1.5 h electrophoretic separation.

Examination of the pooled membranes by electron microscopy revealed vesicles of different sizes for the A peak, with a large amount of cell debris, as shown in Plate 1(a). This contrasts with the B peak, shown in Plate 1(b), which contained microvillar-brush-border vesicles.

To determine whether the vesicles were osmotically active, the 20 min uptake of 0.06 mM-proline was examined by using membranes suspended in solutions of different osmolarity as shown in Fig. 3. Intravesicular size was decreased by increasing the medium osmolarity with sucrose, an impermeant solute. Proline uptake by vesicles from both the A and B peaks was inversely proportional to medium osmolarity when examined over the range 300–1100 mosm. Extrapolation of the lines in Fig. 3 to their y -intercepts, where at an infinitely high medium osmolarity there is presumably no intravesicular space, informs on the amount of substrate that binds to the membranes. These determinations indicate that for the A population, 30.7% of the total proline uptake at a normal medium osmolarity of 300 mosm is due to binding,

Fig. 3. *Effect of medium osmolarity on the uptake of proline by renal brush-border vesicles*

Membranes separated by free-flow electrophoresis designated 'A' (○) and 'B' (●), and these isolated by centrifugation alone (▲) were allowed to incubate for 30 min in medium containing buffer II and 100 mM-NaCl plus various concentrations of sucrose to change the osmolarity of the incubation medium. [¹⁴C]Proline was then added and incubated with the vesicles for 20 min, after which the suspension was filtered and radioactivity was determined as described in the text. Each population of membranes contained 300 mosm buffer and proline uptake measured in 300 mosm incubation medium was assigned a value of 100%. Absolute uptake, in nmol/mg of protein, for the 100% values was 0.11 for the 'A' and 0.12 for the 'B' membranes, and 0.76 for the membranes prepared by centrifugation alone. Results shown are means of duplicate determinations normalized to these values. Relative osmolarity is defined as 300 mosm = 1 = isosmolar when proline uptake is measured in 300 mosm incubation buffer in membranes containing 300 mosm buffer.

EXPLANATION OF PLATE 1

Electron micrographs of negatively stained wet preparations of the A peak (a; magnification $\times 42\,000$) and the B peak (b; magnification $\times 42\,000$) prepared from newborn-rat kidney by centrifugation and free-flow electrophoresis
The scale marker represents $1\ \mu\text{m}$.

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(facing p. 212)

whereas for B this is equal to only 7.4% of total proline uptake. Thus, the A membranes bind more than four times as much proline as do those of B. Also shown in Fig. 3 are the results of similar studies with newborn-rat renal brush-border membranes prepared by homogenization and centrifugation alone (Goldmann *et al.*, 1976). Proline binding to this preparation is equal to 60% of total proline uptake at 20 min. The continued time-dependent uptake of proline previously reported (Goldmann *et al.*, 1979) was largely due to proline binding rather than to transport into the vesicles.

The B membranes enriched in alkaline phosphatase, showing little contamination by basolateral membranes and intracellular organelles, demonstrating osmotic activity and exhibiting low substrate binding, thus provide a suitable model for the study of solute transport by renal brush-border membranes from newborn rats. The time-dependent uptake of proline by renal brush-border-membrane vesicles from newborn rats is shown in Fig. 4. The presence of an inwardly directed 100 mM-NaCl gradient slightly enhanced early proline uptake, when compared with proline uptake by vesicles that were NaCl-equilibrated by pre-incubation for 20 min before the start of uptake determinations. Proline uptake reaches equilibrium by 10 min and remains

Fig. 4. Time course of 0.06 mM-proline uptake by renal brush-border-membrane vesicles prepared from newborn rats

Proline uptake in the presence of an inwardly directed 100 mM-NaCl gradient (●) was significantly greater at 0.5 min ($P < 0.1$) and 1 and 3 min ($P < 0.05$) when compared with uptake at the corresponding times by vesicles that were NaCl-equilibrated (○) by pre-incubation for 20 min before the start of uptake determinations. Values shown are means $\pm$ S.E.M. of at least five determinations.

constant for 60 min under Na^+ -gradient or Na^+ -equilibrated conditions. The intravesicular volume of the B-peak vesicles, as determined by the 20 min uptake of 0.06 mM-proline is approx. $2 \mu\text{l}/\text{mg}$ of vesicle protein.

Discussion

Goldmann *et al.* (1976) described a method for the preparation of purified brush-border membranes isolated from newborn-rat kidney by differential centrifugation. Our present method isolated brush-border membranes from newborn-rat kidney using differential centrifugation followed by free-flow electrophoresis. Free-flow electrophoresis when used to separate adult renal membranes results in two major peaks of membranes, one enriched in alkaline phosphatase and the other in $(\text{Na}^+ + \text{K}^+)$ -stimulated ATPase, indicating the presence of brush-border and basolateral membranes respectively (Heidrich *et al.*, 1972). However, free-flow electrophoresis resolved our newborn-rat kidney preparation into two populations of brush-border membranes with one peak showing the activity of both alkaline phosphatase and $(\text{Na}^+ + \text{K}^+)$ -stimulated ATPase, the basolateral membrane marker enzyme. The enzyme profile of this peak, designated 'A', suggests this is a mixture of separate brush-border and basolateral membrane fragments or a population of membranes with attributes of both types of renal cell membranes. This may be due to the cell membrane of newborn-rat kidney being sheared during homogenization such that some brush-border membranes have attached fragments of basolateral membranes or that the immature renal cell membranes contain both of these enzymes before complete differentiation.

The enzyme composition of the A peak and the preparation described by Goldmann *et al.* (1976) are quite similar, except for the presence of $(\text{Na}^+ + \text{K}^+)$ -stimulated ATPase in our membranes. This may be due to our use of a more sensitive method for detection of this enzyme. This, and the similarity of the homogenization and centrifugation steps in both procedures, suggests that Goldmann's preparation contained the membranes present in our A and B peaks, and that by using free-flow electrophoresis an alkaline phosphatase-enriched brush-border membrane preparation, designated 'B', was isolated.

Osmotic-dependency studies with membranes prepared by free-flow electrophoresis show that at 20 min there is 31% proline binding to the A membranes, whereas there is only 7% binding to those in the B peak. This contrasts with the adult-rat vesicle preparation, in which essentially no proline binding to the membranes is observed (Hsu *et al.*, 1982). The different proline uptake characteristics of the newborn-rat preparations may be due to a

binding component, perhaps a protein, which is partially removed from the A peak and almost totally removed from the B peak with our use of free-flow electrophoresis. The B membranes exhibit substrate-binding and enzyme-purity characteristics that are similar to brush-border membrane vesicles prepared from adult rat kidney. We therefore have used this preparation to study solute transport and for comparison with data obtained from vesicles isolated from adult.

The uptake of 0.06 mM-proline by the 'B' vesicles is rapid, reaches equilibrium by 3 min and maintains a steady state for 60 min. In contrast with the findings of McNamara *et al.* (1976) using adult-rat brush-border vesicles, we did not detect an overshoot with an Na^+ -gradient, but rather a faster rise to equilibrium compared with the Na^+ -equilibrated state. This differs from results with vesicles prepared from newborn-rat kidney by centrifugation alone that showed proline uptake increasing with time, not reaching a steady state by 60 min when the uptake was the same as the adult at 2 min (Goldmann *et al.*, 1979). The reason for this continuous uptake was not apparent from the data, which indicated little proline binding at 2 min. We now present evidence that shows that in vesicles prepared from newborn-rat kidney by centrifugation alone, almost 60% of the proline uptake measured at 20 min is due to binding. The continued time-dependent uptake of proline by these membranes observed by Goldmann *et al.* (1979) therefore resulted from proline binding rather than transport into the vesicles.

The lack of an Na^+ -stimulated overshoot in our newborn-rat preparation does not necessarily indicate a primary change of proline transport. It may be due to secondary changes such as a more rapid dissipation of the Na^+ gradient or to a reduced rate of Na^+ entry in vesicles prepared from newborn rats. Age-related changes in Na^+ transport in vesicles would be consistent with decreased renal sodium re-absorption seen in newborns (Rodriguez-Soriano *et al.*, 1982). Further studies examining the transport of both Na^+ and Na^+ -dependent solutes using vesicles prepared from newborn rats are needed to clarify these issues.

Previous studies of proline transport have employed both renal cortical slices and isolated renal tubules and have shown that the entry of proline is the same in both adult and newborn. The differences between newborn and adult renal amino acid uptake are not due to altered rates of influx but to slower rates of substrate efflux from the renal cortical cells. Although there is no Na^+ -dependent proline overshoot in newborn-rat vesicles the data from renal tubules and slices suggest that the entry parameters describing proline uptake are unaffected in intact cells. Other cellular factors must therefore

be involved to compensate for the decreased brush-border membrane transport of proline, as suggested by the absence of an Na^+ -stimulated proline overshoot in the newborn-rat vesicles. Transport studies on membrane vesicles are performed under very special experimental conditions, and the exact nature of the relationship of these data to the physiological function of the renal proximal-tubule cell remains to be determined.

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